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(54) Title: VIRULENCE GENES, PROTEINS, AND THEIR USE

(57) Abstract: A series of genes from *Brucella spp* are shown to encode products which are implicated in virulence. The identification of these genes therefore allows attenuated microorganisms to be produced. Furthermore, the genes or their encoded products can be used in the manufacture of vaccines for therapeutic application.

VIRULENCE GENES, PROTEINS, AND THEIR USE

Field of the Invention

This invention relates to virulence genes and proteins, and their use. More particularly, it relates to genes and proteins/peptides obtained from *Brucella* species, and their use in therapy and in screening for drugs.

Background of the Invention

Members of the Gram-negative genus *Brucella* cause Brucellosis, an infectious disease of animals that is transmissible to humans. Brucellosis in humans is sometimes called Malta fever or undulant fever and can occur in adults, children or neonates. Brucellosis can result in either an acute or chronic disease and can manifest as a variety of symptoms including arthritis, enteric fever, meningitis, encephalitis, infective endocarditis, myocarditis and cutaneous lesions, as well as giving rise to heamatological abnormalities.

There are currently six species of *Brucella* identified; *B. melitensis, B. suis, B. neotomae, B. ovis, B. canis* and *B. abortus*. The principal hosts of *B. melitensis* are small ruminants (goats and sheep), although infections of cattle are also widespread.

B. melitensis is the most virulent species of the brucellae identified to date, based on the ability to cause infections in humans and cattle. *B. melitensis* infections in animals can result in abortion, sterility and decreased milk production in females, and epididymitis and orchitis in males. It would be desirable to provide means for treating or preventing conditions caused by *Brucella* species in animals and humans e.g. by immunisation.

Summary of the Invention

The present invention is based on the discovery of virulence genes in *B. melitensis*.

According to a first aspect of the invention, a peptide of the invention is encoded by an operon including any of the nucleotide sequences identified herein as SEQ ID NOS. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 32 and 33 of *B. melitensis*, or a homologue thereof in a

Gram-negative bacterium, or a functional fragment thereof, for therapeutic or diagnostic use.

The peptides may have many therapeutic uses for treating *Brucella* infections, including use in vaccines for prophylactic application.

According to a second aspect, a polynucleotide encoding a peptide defined above, may also be useful for therapy or diagnosis.

According to a third aspect, the genes that encode the peptides may be utilised to prepare attenuated microorganisms. The attenuated microorganisms will usually have a mutation that disrupts the expression of one or more of the genes identified herein, to provide a strain that lacks virulence. These microorganisms will also have use in therapy and diagnosis.

According to a fourth aspect, the peptides, genes and attenuated microorganisms according to the invention may be used in the treatment or prevention of a condition associated with infection by *Brucella* or Gram-negative bacteria.

Description of the Invention

The present invention is based on the discovery of genes which encode peptides which are implicated in virulence. The peptides and genes of the invention are therefore very useful for the preparation of therapeutic agents to treat infection. It should be understood that references to therapy also include preventative treatments, e.g. vaccination. Furthermore, while the products of the invention are intended primarily for treatment of infections in human patients, veterinary applications are also considered to be within the scope of the invention.

The present invention is described with reference to *Brucella melitensis*. However, all the *Brucella* strains, and many other Gram-negative bacterial strains are likely to include related peptides or proteins having amino acid sequence homology to those identified herein. Organisms likely to contain the peptides include, but are not limited to, *B. suis*, *B. neotomae*, *B. ovis*, *B. canis* and *B. abortus*.

Preferably, the peptides that may be useful in the various aspects of the invention have greater than a 40% similarity with the peptides identified herein. More preferably, the peptides have greater than 60% sequence similarity. Most preferably, the peptides have greater than 80% sequence similarity, e.g. 95% similarity. With regard to the polynucleotide sequences identified herein, homologs that may be useful in the various aspects of the invention may have greater than 40% identity with the sequences identified herein. More preferably, the polynucleotide sequences have greater than 60% sequence identity. Most preferably, the polynucleotide sequences have greater than 80% sequence identity, e.g. 95% identity.

The terms "similarity" and "identity" are known in the art. The use of the term "identity" refers to a sequence comparison based on identical matches between correspondingly identical positions in the sequences being compared. The term "similarity" refers to a comparison between amino acid sequences, and takes into account not only identical amino acids in corresponding positions, but also functionally similar amino acids in corresponding positions. Thus similarity between polypeptide sequences indicates functional similarity, in addition to sequence similarity.

Levels of identity between gene sequences and levels of identity or similarity between amino acid sequences can be calculated using known methods. In relation to the present invention, publicly available computer based methods for determining identity and similarity include the BLASTP, BLASTN and FASTA (Atschul *et al*, J. Molec. Biol., 1990; 215:403-410), the BLASTX program available from NCBI, and the Gap program from Genetics Computer Group, Madison WI. The levels of similarity and identity provided herein, were obtained using the Gap program, with a Gap penalty of 12 and a Gap length penalty of 4 for determining the amino acid sequence comparisons, and a Gap penalty of 50 and a Gap length penalty of 3 for the polynucleotide sequence comparisons.

Having characterised a gene according to the invention, it is possible to use the gene sequence to establish homologies in other microorganisms. In this way, it is possible to determine whether other microorganisms have similar peptides. Sequence homologies may be established by searching in existing databases, e.g. EMBL or GenBank.

Peptides or proteins according to the invention may be purified and isolated by methods known in the art. In particular, having identified the gene sequence, it will be possible to use recombinant techniques to express the genes in a suitable host. Active fragments and homologues can be identified and may be useful in therapy. For example, the peptides or their active fragments may be used as antigenic determinants in a vaccine, to elicit an immune response. They may also be used in the preparation of antibodies, for passive immunisation, or diagnostic applications. Suitable antibodies include monoclonal antibodies, or fragments thereof, including single chain Fv fragments. Methods for the preparation of antibodies will be apparent to those skilled in the art.

The preparation of vaccines based on attenuated microorganisms is known to those skilled in the art. Vaccine compositions can be formulated with suitable carriers or adjuvants, e.g. alum, as necessary or desired, to provide effective immunisation against infection. The preparation of vaccine formulations will be apparent to the skilled person. The attenuated microorganisms may be prepared with a mutation that disrupts the expression of any of the genes identified herein. The skilled person will be aware of methods for disrupting expression of particular genes. Techniques that may be used include insertional inactivation or gene deletion techniques. Attenuated microorganisms according to the invention may also comprise additional mutations in other genes, for example in a second gene identified herein or in a separate gene required for growth of the microorganism, e.g. an *aro* mutation. Attenuated microorganisms may also be used as carrier systems for heterologous antigens, therapeutic proteins or

nucleic acids. In this embodiment, the attenuated microorganisms are used to deliver a heterologous antigen, protein or nucleic acid to a particular site *in vivo*. Introduction of a heterologous antigen, peptide or nucleic acid into an attenuated microorganism can be carried out by conventional techniques, including the use of recombinant constructs, e.g. vectors, which comprise polynucleotides that express the heterologous antigen or therapeutic protein, and also include suitable promoter sequences. Alternatively, the gene that encodes the heterologous antigen or protein may be incorporated into the genome of the organism and the endogenous promoters used to control expression.

More generally, and as is well known to those skilled in the art, a suitable amount of an active component of the invention can be selected, for therapeutic use, as can suitable carriers or excipients, and routes of administration. These factors would be chosen or determined according to known criteria such as the nature/severity of the condition to be treated, the type of health of the subject etc.

In a separate embodiment, the products of the invention may be used in screening assays for the identification of potential antimicrobial drugs or for the detection for virulence. Routine screening assays are known to those skilled in the art, and can be adapted using the products of the invention in the appropriate way. For example, the products of the invention may be used as the target for a potential drug, with the ability of the drug to inactivate or bind to the target indicating its potential antimicrobial activity.

The products of the present invention were identified using the following procedure:

In summary, signature-tagged mutagenesis (STM) (Hensel *et al.*, Science 1995; 269: 400-403) was used to identify genes required for the *in vivo* pathogenesis of *Brucella*.

Bacterial strains, plasmids and matings.

B. melitensis 16M Nal^r (Verger *et al.*, Brucella spp. Plasmid, 1993; 29:142-146) was used as the parental strain for all experiments. *B. melitensis* strains were grown on solid or liquid 2YT medium with appropriate antibiotics. The
5 *E. coli* strains used in this study were: S17 λpir [*recA thi pro hsd*R^-M^+ RP4::2-Tc::Mu::Km Tn7 lysogenised with λpir phage] (Miller and Mekalanos, J. Bacteriol., 1988; 170(6): 2575-2583), CC118_λ pir [Δ(*are-leu*) *ara*D Δ*lac*X74 *galE galK phoA20 thi*-1 *rpsE rpoB argE recA*1 lysogenised with λ pir
10 phage] (de Lorenzo *et al.*, J. Bacteriol., 1990; 172:6568-6572), and TOP10 [F^- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lacX*14 *recA*1 *deoR araD*139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^r) *endA*1 *nupG*] (Invitrogen). *E. coli* strains were grown on Luria-Bertani (LB) medium with appropriate antibiotics.
15 Antibiotics were used at the following concentration for *E. coli* and *B. melitensis* : Ampicillin (Amp), 50 μg/ml; Kanamycin (Kan), 50 μg/ml; Nalidixic acid (Nal), 25 μg/ml. A modified minimal medium was used. The plasmids used in this study were : pUTmini-Tn5*Km*2 (Hensel *et al.*, *supra*) and
20 pCR TOPO 2.1 (Invitrogen).

The mutant bank was generated by transferring the mini-Tn5 transposon from *E. coli* Lambda pir strains where it is maintained on a plasmid to the *B. melitensis* chromosome. This is achieved by mating the *E. coli* strains containing
25 signature-tagged pUTminiTn5Km2 with the *B. melitensis* parental strain resulting in transfer of the tagged transposon to the *Brucella* chromosome.

Matings were performed by mixing equal volumes (20 μl) of liquid cultures of *E. coli* S17 donor cells (OD 0.6) and
30 the *B. melitenisis* 16M Nal^r recipient strain (overnight culture) on a 0.22 μm filter. The filter was left for one hour on a LB plate without antibiotics and then transferred onto a 2YT plate containing Kan and Nal. After three days incubation at 37°C, the exconjugates were replicated on 2YT
35 Nal Kan and on 2YT Nal Amp. The Amp-resistant clones (about 4% of the clones) were discarded, the Amp-sensitive clones were transferred into 96 well plates.

Amplification and labelling of DNA tags from signature tagged mutants.

A modified version of the protocol from Holden and Hensel (Methods in Microbiology, 1998; 27:359-370) was used for amplification and labelling of tags. Briefly, 10^4 colonies of signature-tagged mutant bacteria from each input and output pool of 96 individual mutants were recovered from agar plates, resuspended in PBS, centrifuged and genomic DNA from the pelleted bacteria recovered by the CTAB method (Ausubel *et al.*, Current Protocols in Molecular Biology, 1991). Tags were initially amplified by PCR from genomic DNA using the primers SEQ ID NO. 34 and 35.

The amplicons were purified and a fraction was used as target DNA in the second PCR including [32-P]-dCTP to radiolabel the tags. The presence or absence of the individual tags in the input and output pools was shown by hybridising the radiolabelled tags to DNA dot blots of the 96 signature tags, amplified by PCR from the 96 signature-tagged pUTminiTn5Km2 plasmids.

Molecular techniques.

DNA manipulation was performed following standard techniques (Ausubel *et al.*, *supra*). Restriction enzymes were purchased from Roche and primers from Amersham Pharmacia.

Identification of mini-Tn5 insertion sites.

Transposon insertion sites were amplified by arbitrary PCR (O'Toole and Kolter, Mol. Microbiol, 1998; 28:449-461) of genomic DNA isolated from the mini-Tn5 mutants. Arbitrary PCR was also performed on genomic DNA from the wild-type as negative control. The PCR products were cloned into pCR TOPO 2.1 (Invitrogen). The inserts were sequenced using the dye terminator method (Big Dye Terminator kit, Perkin Elmer) with an ABI 377 sequencer. Sequences were analysed by performing searches with the Blastx program (Altschul *et al.*, Nucleic Acids Res, 1997; 25: 3389-3402) against the EMBL and GenBank databases.

Screening of the STM library.

Mutants were each grown at 37°C in 200 μl of 2YT in 96 well microtitre plates with appropriate antibiotics for 48 h. The bacteria were then pooled, centrifuged at 4000 r.p.m. for 10 min and resuspended in 2 ml of 0.9% NaCl. The
5 bacterial suspension was then diluted to a final concentration of 5 x 10^5 cfu in 100 μl of 0.9% NaCl. The number of bacteria was confirmed by plating dilutions on 2YT plates. The bacterial suspension was injected i.p. in five week-old female BALB/c mice. The remaining part of the
10 suspension was plated onto media for DNA isolation. Five days after the infection, animals were sacrificed and the spleens removed aseptically. For recovery of bacteria, the spleens were homogenised in PBS 0.1% Triton X-100 (Roche) and dilutions were plated on 2YT. Plates containing
15 approximately 10^4 colonies were used for DNA extraction (the output pool). Signature tags were then amplified from the genomic DNA by a two-step PCR and stringent hybridisations performed as described above.

Competitive index

20 Mutants identified by STM are attenuated in virulence. The level of attenuation was measured by determining the competitive index (CI).

In competition experiments, mutant (Nal^r, Kan^r) and wild-type bacteria (Nal^r) were grown for 48h in 2YT, then
25 equal amounts of bacteria (about 2.5 x 10^5 each in 100 μl of 0.9% NaCl) were mixed and injected i.p. to mice. Dilutions of the infecting doses were plated on 2YT and 2YT Kan to estimate the ratio of mutant to wild-type bacteria in the inoculum. Mice were sacrificed after 5 days, and the spleen
30 recovered and homogenised. To determine the proportion of mutant to wild-type, dilutions of the spleen homogenate were plated on 2YT and 2YT Kan. The competitive index (CI) was calculated as the proportion of mutant to wild-type bacteria recovered from the animals, divided by the proportion of
35 mutant to wild-type in the inoculum. For *in vitro* growth assays, 5 ml of 2YT was inoculated with the infection dose. The cultures were incubated at 37°C for 48 h with shaking (200 r.p.m.) and serial dilutions of the culture were plated

to media with or without Kan. These experiments were performed in duplicate.

Infection of macrophages and HeLa cells.

Brucella spp can replicate within host cells. To determine if any of the mutants were defective in this property, subconfluent monolayers ($2x10^4$) of murine J774 macrophages or human HeLa cells were inoculated with bacteria diluted to $6x10^6$ CFU ml^{-1} in cell culture medium. Plates were centrifuged for 10 min at 1000 r.p.m. at room temperature and placed in a 5% CO_2 atmosphere at 37°C. After 1 h, wells were washed three times and incubated for 48 h with cell culture medium supplemented with 50 μg ml^{-1} gentamycin. At the end of the infection time, the monolayers were washed 3 times with cell culture medium and treated for 20 min with 200 μl of 0.1% Triton X-100 (Roche) in PBS. Serial dilutions of the lysates were plated onto 2YT plates for determination of CFU. Each infection was performed in triplicate.

The following Examples illustrate the invention.

Example 1

A first mutant was identified and the nucleotide sequence immediately following the transposon insertion was cloned. The sequence is shown as SEQ ID NO. 1.

A translation from this sequence is shown as SEQ ID NO. 2.

The nucleotide sequence shows 59.4% identity from nucleotide 1-430 to the *cysI* gene of *Pseudomonas aeruginosa* at nucleotides 1109-1539 of the latter (EMBL accession number AF026066). The amino acid sequence shows 49.5% identity from amino acid 3-103 to amino acids 360-460 of the *Pseudomonas aeruginosa* CysI.

This demonstrates that the disrupted gene is at least partially identical to the *cysI* gene of *Pseudomonas aeruginosa*. The CysI gene has a putative function as a sulphite reductase (TrEMBL accession number 031037).

In the test for attenuation of virulence, the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0.000293 (mean CI from 2 mice).

As the *cysI* genes in *Salmonella typhimurium* and *E. coli* strain B are transcribed as part of an operon with the *cysH* gene, it is possible that this attenuation is due to a polar effect on a presumed *cysH* gene in *Brucella melitensis*.

5 Example 2

A second mutant was identified, and the nucleotide sequence immediately following the transposon insertion was cloned. The sequence is shown as SEQ ID NO. 3. A translation from this sequence is shown in SEQ ID NO. 4.

10 The nucleotide sequence shows 63.3% identity from nucleotide 1-392 to the *mgtB* gene of *Salmonella typhimurium* at nucleotides 3531-3922 of the latter (EMBL accession number M57715). The amino acid sequence shows 44.3% identity from amino acid 1-131 to amino acids 672-802 of the 15 *Salmonella typhimurium mgtB*.

This demonstrates that the disrupted gene is at least partially identical to the *mgtB* gene of *Salmonella typhimurium*. This gene has a putative function as a magnesium transport ATPase (SwissProt accession number 20 P22036).

The amino acid sequence also shows 39.8% identity from amino acid 1-118 to the *Mgt*A protein of *E. coli* K12 (SwissProt accession number P39168) from amino acid 663-780 of the latter.

25 In the test for attenuation of virulence the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0.00189 (mean CI from 2 mice).

The mutant was also tested for invasion of macrophages and HeLa cells, and was found to be attenuated in both.

30 Example 3

A further mutant was identified, and the nucleotide sequence immediately following the transposon insertion was cloned. The sequence is shown as SEQ ID NO. 5. A translation from this sequence is shown as SEQ ID NO. 6.

35 The nucleotide sequence shows 63.2% identity from nucleotide 1-109 to the *y4oU* gene of *Rhizobium sp.* strain NGR234 (EMBL accession number AE000089) at nucleotides 5503-5611 of the latter. The amino acid sequence shows 36.4%

identity from amino acid 1-122 to amino acid 7-126 of the *Rhizobium sp.* Y4oU protein (SwissProt accession number P55606).

This demonstrates that the disrupted gene is at least partially identical to the *y4oU* gene of *Rhizobium sp.* strain NGR234.

In the test for attenuation of virulence, the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0.00362 (mean CI from 2 mice).

As the gene is potentially transcribed as part of an operon with the genes *y4oV* and *y4oW*, it is possible that this attenuation is due to a polar effect on presumed *y4oV* and/or *y4oW* genes.

The mutant was also tested for growth in macrophages and found to be attenuated.

Example 4

A further mutant was identified, and the nucleotide sequence immediately following the transposon insertion was cloned. The sequence is shown as SEQ ID NO. 7. A translation from this sequence is shown as SEQ ID NO. 8.

The nucleotide sequence shows 72.9% identity from nucleotide 46-300 to the *cysK* gene of *Rhodobacter sphaeroides* (EMBL accession number AF004296) at nucleotides 2969-3223 of the latter. The amino acid sequence shows 75.3% identity from amino acid 1-99 to amino acids 190-286 of the *Rhodobacter sphaeroides CysK*.

This demonstrates that the disrupted gene is at least partially identical to the *cysK* gene of *Rhodobacter sphaeroides*. This gene has a putative function as an O-acetylserine(thiol)lyase.

In the test for attenuation of virulence the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0.000214 (mean CI from 2 mice).

The mutant was also attenuated in both macrophages and HeLa cells.

Example 5

A further mutant was identified, and the nucleotide sequence immediately following the transposon insertion was

cloned. The sequence is shown as SEQ ID NO. 9. A translation from this sequence is shown as SEQ ID NO. 10.

The nucleotide sequence shows 69.4% identity from nucleotide 1-98 and 59.0% identity from nucleotides 315-375 to the *metH* gene of *E. coli* at nucleotides 4013-4110 and 3738-3798 of the latter (EMBL accession number AE000475). The amino acid sequence shows 37.7% identity from amino acid 11-79 to amino acids 854-922 of the *E. coli* MetH.

This demonstrates that the disrupted gene is at least partially identical to the *metH* gene of *E. coli* K12. This gene has a putative function as a homocysteine-NS-methyltetrahydrofolate transmethylase.

The amino acid sequence also shows 34.4% identity from amino acid 11-74 to the human 5-methyltetra-hydrofolate homocysteine methyltransferase (SwissProt accession number Q99707) from amino acids 881-944 of the latter.

In the test for attenuation of virulence the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0.000225 (mean CI from 2 mice).

The mutant was also attenuated in both macrophages and HeLa cells.

Example 6

A further mutant was identified, and the nucleotide sequence immediately following the transposon insertion was cloned. The sequence is shown as SEQ ID NO. 11. A translation of this sequence is shown as SEQ ID NO. 12.

This nucleotide sequence shows 67.3% identity from nucleotide 1-101 to the *yqjG* gene of *E. coli* K12 (EMBL accession number U188997) at nucleotides 32481-32581 of the latter. The amino acid sequence shows 75% identity from amino acid 1-32 to amino acid 236-267 of the E. coli *yqjG* (SwissProt accession number P42620).

This demonstrates that the disrupted gene is at least partially identical to the *yqjG* gene of *E. coli* K12.

The amino acid sequence also shows 87.9% identity from amino acid 1-33 to amino acid 235-267 of an hypothetical protein (dbj) from *Synechocystis* sp. strain PCC6803 (TrEMBL accession number P74752).

In the test for attenuation of virulence the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0,00109 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

Example 7

A further mutant was identified, and the nucleotide sequence immediately following the transposon insertion was cloned. The sequence is shown as SEQ ID NO. 13. A translation from this sequence is shown as SEQ ID NO. 14.

This nucleotide sequences shows 54.5% identity from nucleotide 278-545 to the *dnaJ* gene of *Thermus thermophilus* (EMBL accession number L57504) at nucleotide 3072-3329 of the latter. The amino acid sequence shows 41.5% identity from amino acid 1-132 to amino acid 85-209 of the *Thermus thermophilus* DnaJ.

This demonstrates that the disrupted gene is at least partially identical to the *dnaJ* gene of *Thermus thermophilus*. This gene has a putative role as a chaparone (SwissProt accession number Q56237).

The amino acid sequence also shows homology to a number of other proteins belonging to the protein family of DnaJ proteins (accession number PF00226). This family includes the DnaJ protein from *Salmonella typhimurium* (SwissProt accession number Q60004) and of *Mycobacterium leprae* (SwissProt accession number Q02605).

In the test for attenuation of virulence the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0.00004 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

Example 8

A further mutant was identified, and the nucleotide sequence immediately following the transposon insertion was cloned. The sequence is shown as SEQ ID NO. 15. A translation of this sequence from nucleotides 1 to 537 is shown as SEQ ID NO. 16.

This is a previously unknown gene and has been termed *bru1*.

In the test for attenuation of virulence the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0.000471 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

Example 9

A further mutant was identified, and the nucleotide sequence immediately following the transposon insertion was cloned. The sequence is shown as SEQ ID NO. 17. A translation from this sequence is shown as SEQ ID NO. 18.

The gene shows 100% identity to nucleotides 1372-1491 of a sequence from *Brucella abortus* (AF011895). This sequence contains the *ccrM* gene known to encode an adenine DNA methyl transferase. However, the region of AF011895 with homology to the gene is located downstream of the *ccrM* gene, and no open reading frame has currently been ascribed to this region. This shows that *Brucella abortus* also contains a gene that is at least partially identical to the gene of *B. melitensis*.

In the test for attenuation of virulence the mutated microorganism was shown to be attenuated with a competitive index (CI) of 0.0000555 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

Example 10

A further mutant was identified, and the nucleotide sequence on either side of the transposon insertion was cloned. The sequence at one end is shown as SEQ ID NO. 19, and the sequence at the other end is shown as SEQ ID NO. 21.

A translation from SEQ ID NO. 19 is shown as SEQ ID NO. 20. A translation from SEQ ID NO. 21 is shown as SEQ ID NO. 22.

The 641 nucleotide sequence (SEQ ID NO. 19) shows 57.8% identity from nucleotide 11-641 to the *flhS* gene of *Paracoccus denitrificans* (EMBL accession number AJ223460) at nucleotides 690-1308 of the latter. The 403 nucleotide

sequence (SEQ ID NO. 21) shows 61.1% identity from nucleotide 59-233 to the *flhR* gene of *Paracoccus denitrificans* (EMBL accession number AJ223460). The 213 amino acid sequence (SEQ ID NO. 20) shows 36.8% identity
5 from amino acid 1-210 to amino acid 221-426 of the *Paracoccus denitrificans* FlhS protein (TrEMBL accession number O54012). The 89 amino acid sequence (SEQ ID NO. 22) shows 44.6% identity from amino acid 18-82 to amino acid 166-230 of the *Paracoccus denitrificans* FlhR (TrEMBL
10 accession number O54014).

This demonstrates that the disrupted genes are at least partially identical to the *flhS* and *flhR* genes of *Paracoccus denitrificans*.

The 213 amino acid sequence also shows 33.3% identity
15 from amino acid 1-210 to amino acid 400-613 of the VsrB protein of *Pseudomonas solanacearum* (TrEMBL accession number Q52582).

The 89 amino acid sequence also shows 56.1% identity from amino acid 25-81 to amino acid 157-213 the VsrC protein
20 of *Pseudomonas solanacearum* (TrEMBL accession number Q45415).

As the *flhS* and *flhR* genes of *Paracoccus denitrificans* are potentially transcribed as part of an operon with a putative open reading frame *orf2*, it is possible that this
25 attenuation is due to a polar effect on a presumed gene homologous to *orf2* or the actual insertion of the mini-Tn5 transposon in a presumed *orf2*.

In the test for attenuation of virulence the mutated microorganism was shown to be attenuated with a competitive
30 index (CI) of 0.00052 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

Example 11

A further mutant was identified, and the nucleotide
35 sequence either side of the transposon insertion was cloned. The sequence at one end is shown as SEQ ID NO. 23, and the sequence at the other end is shown as SEQ ID NO. 25.

A translation from SEQ ID NO. 23 is shown as SEQ ID NO. 24. This predicted protein shows 66% identity to the Rbsc-2 protein of *A. fulgodus* at amino acids 27 to 44.

A translation from SEQ ID NO. 25 is shown as SEQ ID NO. 26. This sequence shows 56.3% identity to the *rbsc-2* gene of *A. fulgodus* at nucleotides 2873 to 3007 and 37% identity to amino acids 96 to 149.

This demonstrates that the disrupted gene is at least partially identical to the *rsbc2* gene of *A. fulgidus* (EMBL accession number AJ224684). The *rbsc*2 gene encodes a probable ribose ABC transporter permease protein.

In the test for attenuation of virulence, the mutated microorganisms was shown to be attenuated with a competitive index (CI) of 0.00638 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

Example 12

A further mutant was identified, and the nucleotide sequence either side of the transposon insertion was cloned. The sequence at one end is shown as SEQ ID NO. 27, and the sequence at the other end is shown as SEQ ID NO. 29.

A translation of SEQ ID NO. 27 is shown as SEQ ID NO. 28. A translation of SEQ ID NO. 29 is shown as SEQ ID NO. 30.

The 360 nucleotide sequence (SEQ ID NO. 27) shows 54.8% identity from nucleotide 17-359 to the *ugpA* gene of *E. coli* K12 (EMBL accession number X13141) at nucleotides 1823-2165 of the latter. The 375 nucleotide sequence (SEQ ID NO. 29) shows 61.4% identity from nucleotide 62-372 to the *ugpB* gene of *E. coli* K12 at nucleotides 1321-1631 of the latter. The 119 amino acid sequence (SEQ ID NO. 28) shows 43.6% identity from amino acid 1-117 to amino acid 19-135 of the UgpA protein of *E. coli* K12 (SwissProt accession number P10905). The 112 amino acid sequence (SEQ ID NO. 30) shows 48.1% identity from amino acid 1-117 to amino acid 19-135 of the UgpA protein of *E. coli* K12 (SwissProt accession number P10904).

This demonstrates that the disrupted gene is at least partially identical to the *ugp* operon of *E. coli* K12.

As the *ugpA* and *ugpB* genes are transcribed as part of an operon with the *ugpC* and *ugpE* gene, it is possible that this attenuation is due to a polar effect on a presumed *ugpC* or/and *ugpE* gene in *Brucella melitensis*.

The mutant was also attenuated in macrophages and HeLa cells.

Example 13

A further mutant was identified, and the nucleotide sequence following the transposon insertion was cloned. The nucleotide sequence is shown as SEQ ID NO. 31.

The sequence shows 80% homology to the *mtgtA* gene of H. *influenzae*.

In the test for attenuation of virulence, the mutant was shown to be attenuated with a competitive index (CI) of 0.0002 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

Example 14

A further mutant was identified, and the nucleotide sequence following the transposon insertion was cloned. The nucleotide sequence is shown as SEQ ID NO. 32.

The sequence shows 40% homology to a gene of unknown function in S. *meliloti*.

In the test for attenuation of virulence, the mutant was shown to be attenuated with a competitive index of 0.0000373 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

Example 15

A further mutant was identified, and the nucleotide sequence following the transposon insertion was cloned. The nucleotide sequence is shown as SEQ ID NO. 33.

The sequence has no homologies to other known genes.

In the test for attenuation of virulence, the mutant was shown to be attenuated with a competitive index of 0.000277 (mean CI from 2 mice).

The mutant was also attenuated in macrophages and HeLa cells.

CLAIMS

1. A peptide encoded by an operon including any of the nucleotide sequences identified herein as SEQ ID NOS. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 32 and 33 of *B. melitensis*, or a homologue thereof in a Gram-negative bacterium, or a functional fragment thereof, for therapeutic or diagnostic use.

2. A peptide according to claim 1, wherein the homologue has at least 40% sequence similarity.

3. A peptide according to claim 1 or claim 2, wherein the homologue has at least 60% sequence similarity.

4. A peptide according to any of claims 1 to 3, wherein the homologue has at least 90% sequence similarity.

5. A peptide according to claim 1, comprising the amino acid sequences identified herein as SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 30.

6. A polynucleotide encoding a peptide according to any preceding claim, for therapeutic or diagnostic use.

7. A polynucleotide comprising a nucleotide sequence as defined in claim 1, for therapeutic or diagnostic use.

8. A host transformed to express a peptide according to any of claims 1 to 5.

9. An attenuated microorganism comprising a mutation that disrupts the expression of any of the nucleotide sequences defined in claim 1.

10. A microorganism according to claim 9, wherein the mutation is insertional inactivation or a gene deletion.

11. A microorganism according to claim 9 or claim 10, wherein the microorganism is *Brucella* species.

12. A microorganism according to any of claims 9 to 11, for therapeutic or diagnostic use.

13. A microorganism according to any of claims 9 to 12, comprising a mutation in a further nucleotide sequence.

14. A microorganism according to any of claims 9 to 13, comprising a heterologous antigen, therapeutic peptide or nucleic acid.

15. A vaccine comprising a peptide according to any of claims 1 to 5, or the means for its expression.

16. A vaccine comprising a microorganism according to any of claims 9 to 14.

17. Use of a product according to any of claims 1 to 7, for screening potential drugs or for the detection of virulence.

5 18. Use of a product according to any of claims 1 to 14, for the manufacture of a medicament for use in the treatment or prevention of a condition associated with infection by *Brucella* or Gram-negative bacteria.

19. Use according to claim 18, wherein the condition is
10 Brucellosis.

20. An antibody, raised against a product according to any of claims 1 to 7.

21. Use of a product according to any of claims 1 to 14, in a screening assay for the identification of an
15 antimicrobial drug.

SEQUENCE LISTING

<110> L'Unite de Recherche en Biologie Moleculaire ....

<120> VIRULENCE GENES, PROTEINS, AND THEIR USE

<130> REP06268WO

<140> (not yet known)
<141> 2000-11-01

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gtgatgcgga atttgcaagc tgggtcgcgc gcaatgtcac cggggccaag catcccgatt 180

atgccgtcgt caccatctcg ctgaagcctg tcggcgggca ctcccggcga tgccagtgct 240

gaacagatga act ggt gcc gat gtg gcg caa acc tgt tcc ttc gat gaa     289
           Thr Gly Ala Asp Val Ala Gln Thr Cys Ser Phe Asp Glu
             1               5                  10

atc cgg gtg agc cac gag cag aac ctc gtg ctg cca cat gtg gcc aag    337
Ile Arg Val Ser His Glu Gln Asn Leu Val Leu Pro His Val Ala Lys
     15                  20                  25

gcc gat ctg ccg gca gtc tac gac cgc ctg caa tcg ggc gga ctg gtg    385
Ala Asp Leu Pro Ala Val Tyr Asp Arg Leu Gln Ser Gly Gly Leu Val
 30                  35                  40                  45

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Thr Pro His Ala Gly Leu Ile Thr Asp Ile Ile Ala Cys Pro Gly Leu
                 50                  55                  60
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att tcc gag cgc ttc gcc gat ctg gac cgt cag ctt gaa atc ggc gat      529
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Pro Ala Val Tyr Asp Arg Leu Gln Ser Gly Gly Leu Val Thr Pro His
        35                  40                  45

Ala Gly Leu Ile Thr Asp Ile Ile Ala Cys Pro Gly Leu Asp Tyr Cys
    50                  55                  60

Ala Leu Ala Asn Ala Arg Ser Ile Pro Val Ala Gln Arg Ile Ser Glu
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atc gtg ctg gac gaa ggc gtg gtc acg nac cgc gag acc ttc ggg nat 96
Ile Val Leu Asp Glu Gly Val Val Thr Xaa Arg Glu Thr Phe Gly Xaa
20 25 30

atg atg aac tnc ctc atc atg tgc ccc agc ttg aac ttt gcg aan gtc 144
Met Met Asn Xaa Leu Ile Met Cys Pro Ser Leu Asn Phe Ala Xaa Val
35 40 45

tgc tac ngg atg gcg gca anc gca ttg atc cag tgc ntt acn atg cnc 192
Cys Tyr Xaa Met Ala Ala Xaa Ala Leu Ile Gln Cys Xaa Xaa Met Xaa
50 55 60

ntc att cac caa ctg ntc cag aac ctg atg tac nat atc tcc cag gtn 240
Xaa Ile His Gln Leu Xaa Gln Asn Leu Met Tyr Xaa Ile Ser Gln Val
65 70 75 80

gag ctg tcg agg aac aag atg gac aaa gga gat tct ggc caa gcc ccg 288
Glu Leu Ser Arg Asn Lys Met Asp Lys Gly Asp Ser Gly Gln Ala Pro
85 90 95

ccg atg gga tgc cag aaa cat gng cgc ttc atg atc tgg acg gct ccg 336
Pro Met Gly Cys Gln Lys His Xaa Arg Phe Met Ile Trp Thr Ala Pro
100 105 110

acc tcg tcg atc atg gac atn cca cnt ttg cgc tna ntg ncg tan gcg 384
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            20                  25                  30

Met Met Asn Xaa Leu Ile Met Cys Pro Ser Leu Asn Phe Ala Xaa Val
        35                  40                  45

Cys Tyr Xaa Met Ala Ala Xaa Ala Leu Ile Gln Cys Xaa Xaa Met Xaa
    50                  55                  60

Xaa Ile His Gln Leu Xaa Gln Asn Leu Met Tyr Xaa Ile Ser Gln Val
65                  70                  75                  80

Glu Leu Ser Arg Asn Lys Met Asp Lys Gly Asp Ser Gly Gln Ala Pro
                85                  90                  95

Pro Met Gly Cys Gln Lys His Xaa Arg Phe Met Ile Trp Thr Ala Pro
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  1               5                   10                  15

gtc atc gaa agg gct ggc ggc tca ccg gtg atg atc gat gtc agc att    97
Val Ile Glu Arg Ala Gly Gly Ser Pro Val Met Ile Asp Val Ser Ile
            20                  25                  30

ntg ggc aat ccg cct tat gag ccg gct tat tcc aag cat gat gtg gct    145
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Xaa Gly Asn Pro Pro Tyr Glu Pro Ala Tyr Ser Lys His Asp Val Ala
        35                  40                  45

gaa gcc gcc gga acg acg gtt cag gcg att atc gac agc ggc gat gaa      193
Glu Ala Ala Gly Thr Thr Val Gln Ala Ile Ile Asp Ser Gly Asp Glu
    50                  55                  60

cat agc gcc atg gcc ctg atg gcc gaa ggg gcg acg gct ctc gtt cgt      241
His Ser Ala Met Ala Leu Met Ala Glu Gly Ala Thr Ala Leu Val Arg
65                  70                  75                  80

ggc ctt tcg caa cgc ggc cag gtc gat ggc atg att gcg ctt ggc ggt      289
Gly Leu Ser Gln Arg Gly Gln Val Asp Gly Met Ile Ala Leu Gly Gly
                85                  90                  95

tcg ctt ggc acg gac ctt gcg ctg gat att gcg gcg atc ctg ccg ctt      337
Ser Leu Gly Thr Asp Leu Ala Leu Asp Ile Ala Ala Ile Leu Pro Leu
            100                 105                 110

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            20                  25                  30

Xaa Gly Asn Pro Pro Tyr Glu Pro Ala Tyr Ser Lys His Asp Val Ala
        35                  40                  45

Glu Ala Ala Gly Thr Thr Val Gln Ala Ile Ile Asp Ser Gly Asp Glu
    50                  55                  60

His Ser Ala Met Ala Leu Met Ala Glu Gly Ala Thr Ala Leu Val Arg
65                  70                  75                  80

Gly Leu Ser Gln Arg Gly Gln Val Asp Gly Met Ile Ala Leu Gly Gly
                85                  90                  95
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   1               5                   10                  15

aaa att gct ctg gcg gac ccg cat ggc gcg gcg ctt cac gcc ttc tat      95
Lys Ile Ala Leu Ala Asp Pro His Gly Ala Ala Leu His Ala Phe Tyr
                20                  25                  30

acg acc ggc gaa ttg aag gca gaa ggc gat tcg atc acc gaa ggc atc     143
Thr Thr Gly Glu Leu Lys Ala Glu Gly Asp Ser Ile Thr Glu Gly Ile
            35                  40                  45

ggg cag ggg cgc atc acc gcc aat ctt gaa ggc ttc acc cct gat ttt     191
Gly Gln Gly Arg Ile Thr Ala Asn Leu Glu Gly Phe Thr Pro Asp Phe
        50                  55                  60

tcc tac cag att ccc gat gcg gag gcg ctc gat atc ctg ttt gcc ctc     239
Ser Tyr Gln Ile Pro Asp Ala Glu Ala Leu Asp Ile Leu Phe Ala Leu
    65                  70                  75

gtg gaa gag gag ggg ctt tgc ctc ggc ggc tcg tcc ggc atc aac att     287
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80                  85                  90                  95

gcc ggt gca atc cct gtc tct tgatcagatc tggccgccta gaatcactag        338
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Thr Gly Glu Leu Lys Ala Glu Gly Asp Ser Ile Thr Glu Gly Ile Gly
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Gln Gly Arg Ile Thr Ala Asn Leu Glu Gly Phe Thr Pro Asp Phe Ser
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Tyr Gln Ile Pro Asp Ala Glu Ala Leu Asp Ile Leu Phe Ala Leu Val
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Ser Arg Ala Val Gly Val Val Ser Asn Leu Leu Ser Pro Glu Gly Lys
            20   25   30

cag gcc tat atc gac ggt tta cgc aac gaa tat gcc aag gtg gct gct 145
Gln Ala Tyr Ile Asp Gly Leu Arg Asn Glu Tyr Ala Lys Val Ala Ala
35 40 45

gcc cat gcc cgc aac gag gct gaa aaa cag cgc ctg cca att gcc cgc 193
Ala His Ala Arg Asn Glu Ala Glu Lys Gln Arg Leu Pro Ile Ala Arg
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gcg cgc gcc aat ccg cat cag ctc gac tgg gaa aac tac gag ccg 238
Ala Arg Ala Asn Pro His Gln Leu Asp Trp Glu Asn Tyr Glu Pro
65 70 75

gtgaagccga ccttcaccgg aacgaaggtt tttgaaacct atgatctggc gaaatcgcgc 298

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Tyr Pro

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Pro

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        35                  40                  45

cag gcc gtg ggc gcg gaa aag gtc gag gct atc ttt cca aac ggc aag     192
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    50                  55                  60

cat ctg aaa ata aaa ctg cca aaa ttc gtc gag gat ggc cag acc att     240
His Leu Lys Ile Lys Leu Pro Lys Phe Val Glu Asp Gly Gln Thr Ile
65                  70                  75                  80

cgc ctc aag ggg cag ggt gaa ccg ctg atg ggc ggt acg ccg ggg gac     288
Arg Leu Lys Gly Gln Gly Glu Pro Leu Met Gly Gly Thr Pro Gly Asp
                85                  90                  95

gcg ctg gtg acg atc cgc ttc aag ccg cat tcg cgt ttc cgc ttg gaa     336
Ala Leu Val Thr Ile Arg Phe Lys Pro His Ser Arg Phe Arg Leu Glu
            100                 105                 110

ggc cgc gac gtt cat gtc gac ctt ccg gtc agc att gac gat gcg gtt     384
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ctg ggc ggc aag can gaa agt gga aac tct tgatgggcgc atttcggtga       434
Leu Gly Gly Lys Thr Glu Ser Gly Asn Ser
    130                 135

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                20                  25                  30

Pro Ser Lys Gly Ala Asp Leu Ser Ala Ser Ile Asp Ile Ser Leu Ser
        35                  40                  45

Gln Ala Val Gly Ala Glu Lys Val Glu Ala Ile Phe Pro Asn Gly Lys
    50                  55                  60

His Leu Lys Ile Lys Leu Pro Lys Phe Val Glu Asp Gly Gln Thr Ile
65                  70                  75                  80

Arg Leu Lys Gly Gln Gly Glu Pro Leu Met Gly Gly Thr Pro Gly Asp
                85                  90                  95

Ala Leu Val Thr Ile Arg Phe Lys Pro His Ser Arg Phe Arg Leu Glu
            100                 105                 110

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Leu Gly Gly Lys Thr Glu Ser Gly Asn Ser
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gct tcg cca gat ggt gtt ttc tgc acc gac acc gcc ggt atc gac gcc      95
Ala Ser Pro Asp Gly Val Phe Cys Thr Asp Thr Ala Gly Ile Asp Ala
                20                  25                  30

atc aat atg cat gat ccg cgt ata tcc gtt gcg acc ata cgc aat cac      143
Ile Asn Met His Asp Pro Arg Ile Ser Val Ala Thr Ile Arg Asn His
            35                  40                  45

gcg cgt gtc gaa gcg ggg cag atg gtt gcg acg atc aag atc att ccc      191
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Ala Arg Val Glu Ala Gly Gln Met Val Ala Thr Ile Lys Ile Ile Pro
        50                  55                  60

ttt gcc gtt ccg ggc gtc ctg atc gaa gaa att cgc gca gtc gtg cac     239
Phe Ala Val Pro Gly Val Leu Ile Glu Glu Ile Arg Ala Val Val His
    65                  70                  75

ggc aga ccg ctt tta cag gtc gaa aaa ttc cgg aat atg cag gtc ggc     287
Gly Arg Pro Leu Leu Gln Val Glu Lys Phe Arg Asn Met Gln Val Gly
80                  85                  90                  95

ctc atc cag tcg cgc ctg ccc tcg atc cgc gaa acg gtt ctc gac agg     335
Leu Ile Gln Ser Arg Leu Pro Ser Ile Arg Glu Thr Val Leu Asp Arg
                100                 105                 110

acg ctg gag ctt atg cac aag cgc gta acc cgc aat ggc ggc gcg gtg     383
Thr Leu Glu Leu Met His Lys Arg Val Thr Arg Asn Gly Gly Ala Val
            115                 120                 125

gtc acg caa gag cgc gtc gca cat cat gac gag gcg ctt gcg aca gcc     431
Val Thr Gln Glu Arg Val Ala His His Asp Glu Ala Leu Ala Thr Ala
        130                 135                 140

att gca aag gtt ggc agc gcc tgc gat ctc atc gtc att ttc agt gct     479
Ile Ala Lys Val Gly Ser Ala Cys Asp Leu Ile Val Ile Phe Ser Ala
    145                 150                 155

ttc ggc ggt ggc ccg atg aac cga cat cgg tcc gcg cgc cat cag gcg     527
Phe Gly Gly Gly Pro Met Asn Arg His Arg Ser Ala Arg His Gln Ala
160                 165                 170                 175

cgc cgg ggg tgagatcggt gaagtcgccg cngcgctcgc aatcggacgc            576
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            20                  25                  30
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Asn Met His Asp Pro Arg Ile Ser Val Ala Thr Ile Arg Asn His Ala
        35                  40                  45

Arg Val Glu Ala Gly Gln Met Val Ala Thr Ile Lys Ile Ile Pro Phe
    50                  55                  60

Ala Val Pro Gly Val Leu Ile Glu Glu Ile Arg Ala Val Val His Gly
65                  70                  75                  80

Arg Pro Leu Leu Gln Val Glu Lys Phe Arg Asn Met Gln Val Gly Leu
                85                  90                  95

Ile Gln Ser Arg Leu Pro Ser Ile Arg Glu Thr Val Leu Asp Arg Thr
            100                 105                 110

Leu Glu Leu Met His Lys Arg Val Thr Arg Asn Gly Gly Ala Val Val
        115                 120                 125

Thr Gln Glu Arg Val Ala His His Asp Glu Ala Leu Ala Thr Ala Ile
    130                 135                 140

Ala Lys Val Gly Ser Ala Cys Asp Leu Ile Val Ile Phe Ser Ala Phe
145                 150                 155                 160

Gly Gly Gly Pro Met Asn Arg His Arg Ser Ala Arg His Gln Ala Arg
                165                 170                 175

Arg Gly




<210> 17
<211> 120
<212> DNA
<213> Brucella melitensis

<220>
<221> CDS
<222> (3)..(119)

<400> 17
at atc cgc ctc atc aaa ccc gac cgg gcg atc tac gat cat cac gtc      47
   Ile Arg Leu Ile Lys Pro Asp Arg Ala Ile Tyr Asp His His Val
   1               5                   10                  15

gcc tcg ttc ggc ctc gat ccg gca gca acg ctc ttc atc gat gac acg      95
```

```
Ala Ser Phe Gly Leu Asp Pro Ala Ala Thr Leu Phe Ile Asp Asp Thr
                20                  25                  30

atg cac aat gtg gaa ggt gcg aag c                                    120
Met His Asn Val Glu Gly Ala Lys
            35
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<210> 18
<211> 39
<212> PRT
<213> Brucella melitensis

<400> 18

```
Ile Arg Leu Ile Lys Pro Asp Arg Ala Ile Tyr Asp His His Val Ala
  1               5                  10                  15

Ser Phe Gly Leu Asp Pro Ala Ala Thr Leu Phe Ile Asp Asp Thr Met
            20                  25                  30

His Asn Val Glu Gly Ala Lys
        35
```

<210> 19
<211> 641
<212> DNA
<213> Brucella melitensis

<220>
<221> CDS
<222> (3)..(641)

<400> 19

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tg ctg ggc gtg cgg cgg cgc gga aat ttc gtc gac ctg cag gtg ctc      47
   Leu Gly Val Arg Arg Arg Gly Asn Phe Val Asp Leu Gln Val Leu
     1               5                  10                  15

gac acc ggc atc ggc att gcg cca caa aaa ctg aag ctc gtc ttc cgg      95
Asp Thr Gly Ile Gly Ile Ala Pro Gln Lys Leu Lys Leu Val Phe Arg
                20                  25                  30

gag ttc acg cgg ctc aac gaa ggc atg cgg gaa gcc gaa ggg ctg ggg     143
Glu Phe Thr Arg Leu Asn Glu Gly Met Arg Glu Ala Glu Gly Leu Gly
            35                  40                  45

ctt ggc ctt tcc atc gtg gac cgc atc gcg cgg gtg ctt tcg ctg ccg     191
```

```
Leu Gly Leu Ser Ile Val Asp Arg Ile Ala Arg Val Leu Ser Leu Pro
        50                  55                  60

ctg tcg ctt gcc tca aca ccc ggc aaa ggt acg gtg ttt tcg ctg cgc    239
Leu Ser Leu Ala Ser Thr Pro Gly Lys Gly Thr Val Phe Ser Leu Arg
    65                  70                  75

att ccg gtt tca ggc gag gcg ccg cca gcc gaa gag gtt aaa aac cgt    287
Ile Pro Val Ser Gly Glu Ala Pro Pro Ala Glu Glu Val Lys Asn Arg
80                  85                  90                  95

cgc ggg cct cag cgc gct tcc gag ctt gac ggg ctt ggc gtc ctt tgc    335
Arg Gly Pro Gln Arg Ala Ser Glu Leu Asp Gly Leu Gly Val Leu Cys
                100                 105                 110

atc gac aat gac gtc aat att cta tgc ggc atg gaa acg ctg ctg agc    383
Ile Asp Asn Asp Val Asn Ile Leu Cys Gly Met Glu Thr Leu Leu Ser
            115                 120                 125

ggc tgg ggc tgc aac gtg acc acg ctt cgc agc ggc gcg gag ttg aaa    431
Gly Trp Gly Cys Asn Val Thr Thr Leu Arg Ser Gly Ala Glu Leu Lys
        130                 135                 140

aac ttc tgc gca acc ggc acg gcc gcg cct gcc gtc atc att gcc gat    479
Asn Phe Cys Ala Thr Gly Thr Ala Ala Pro Ala Val Ile Ile Ala Asp
    145                 150                 155

tat cat ctg atc cac gaa aac ggc ctc gac atg atc ggc ttt gcg cgc    527
Tyr His Leu Ile His Glu Asn Gly Leu Asp Met Ile Gly Phe Ala Arg
160                 165                 170                 175

gaa agc ttc aag acc gaa ata ccg gcc atc ctg cta act gcc gac cgc    575
Glu Ser Phe Lys Thr Glu Ile Pro Ala Ile Leu Leu Thr Ala Asp Arg
                180                 185                 190

tcc aag gag gtg cgc cag cgc gcc gag gat gag aac gtc acc gtg ctg    623
Ser Lys Glu Val Arg Gln Arg Ala Glu Asp Glu Asn Val Thr Val Leu
            195                 200                 205

cac aag ccc tgt ctc ttg                                            641
His Lys Pro Cys Leu Leu
        210
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<210> 20
<211> 213
<212> PRT
<213> Brucella melitensis

<400> 20

Leu Gly Val Arg Arg Arg Gly Asn Phe Val Asp Leu Gln Val Leu Asp
1   5   10   15

Thr Gly Ile Gly Ile Ala Pro Gln Lys Leu Lys Leu Val Phe Arg Glu
            20   25   30

Phe Thr Arg Leu Asn Glu Gly Met Arg Glu Ala Glu Gly Leu Gly Leu
        35   40   45

Gly Leu Ser Ile Val Asp Arg Ile Ala Arg Val Leu Ser Leu Pro Leu
    50   55   60

Ser Leu Ala Ser Thr Pro Gly Lys Gly Thr Val Phe Ser Leu Arg Ile
65   70   75   80

Pro Val Ser Gly Glu Ala Pro Pro Ala Glu Glu Val Lys Asn Arg Arg
                85   90   95

Gly Pro Gln Arg Ala Ser Glu Leu Asp Gly Leu Gly Val Leu Cys Ile
            100   105   110

Asp Asn Asp Val Asn Ile Leu Cys Gly Met Glu Thr Leu Leu Ser Gly
        115   120   125

Trp Gly Cys Asn Val Thr Thr Leu Arg Ser Gly Ala Glu Leu Lys Asn
    130   135   140

Phe Cys Ala Thr Gly Thr Ala Ala Pro Ala Val Ile Ile Ala Asp Tyr
145   150   155   160

His Leu Ile His Glu Asn Gly Leu Asp Met Ile Gly Phe Ala Arg Glu
                165   170   175

Ser Phe Lys Thr Glu Ile Pro Ala Ile Leu Leu Thr Ala Asp Arg Ser
            180   185   190

Lys Glu Val Arg Gln Arg Ala Glu Asp Glu Asn Val Thr Val Leu His
        195   200   205

Lys Pro Cys Leu Leu
    210

<210> 21
<211> 403

<212> DNA
<213> Brucella melitensis

<220>
<221> CDS
<222> (1)..(267)

<400> 21
gga ctg ggg gat atc gac ctc gac cac ccg aaa gac ccg gaa atc gaa 48
Gly Leu Gly Asp Ile Asp Leu Asp His Pro Lys Asp Pro Glu Ile Glu
1 5 10 15

ccg ctc att gcg cgg ctg cgc acc ctg acg ccg cag caa acc cgc gtt 96
Pro Leu Ile Ala Arg Leu Arg Thr Leu Thr Pro Gln Gln Thr Arg Val
20 25 30

ctg acc atg ctt gcc gag ggg ctt ctc aac aaa cag atc gct ttt gag 144
Leu Thr Met Leu Ala Glu Gly Leu Leu Asn Lys Gln Ile Ala Phe Glu
35 40 45

ctt ggc gtt tcg gag gca acg gtc aag gcg cat gtt tcc gcc gtg ctg 192
Leu Gly Val Ser Glu Ala Thr Val Lys Ala His Val Ser Ala Val Leu
50 55 60

caa aag ctt ggc gtc gat agc cgc aca cag gcc gtc att ctc ctt tcg 240
Gln Lys Leu Gly Val Asp Ser Arg Thr Gln Ala Val Ile Leu Leu Ser
65 70 75 80

cgt atc ggc agc gac gtt ctg ggc gtt tgagccagct cagctatagg 287
Arg Ile Gly Ser Asp Val Leu Gly Val
85

agctgtcggc ctttgccgct tatcgtgtac tgccttgcgt cgatgccccg tcctggcgtg 347

cgtgatgaaa aagcgagagc atcgagcgca gcgccgctgg ccgcaagggc ttgtgc 403

<210> 22
<211> 89
<212> PRT
<213> Brucella melitensis

<400> 22
Gly Leu Gly Asp Ile Asp Leu Asp His Pro Lys Asp Pro Glu Ile Glu
1 5 10 15

Pro Leu Ile Ala Arg Leu Arg Thr Leu Thr Pro Gln Gln Thr Arg Val
20 25 30

```
Leu Thr Met Leu Ala Glu Gly Leu Leu Asn Lys Gln Ile Ala Phe Glu
        35                  40                  45

Leu Gly Val Ser Glu Ala Thr Val Lys Ala His Val Ser Ala Val Leu
    50                  55                  60

Gln Lys Leu Gly Val Asp Ser Arg Thr Gln Ala Val Ile Leu Leu Ser
65                  70                  75                  80

Arg Ile Gly Ser Asp Val Leu Gly Val
                85
```

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<210> 23
<211> 536
<212> DNA
<213> Brucella melitensis

<220>
<221> CDS
<222> (314)..(520)

<400> 23
ggtttcacgg cgatcatcgt tgcctttctg ggacggctta atccgctggg tgcgattgtg 60

gcgggccttg tgctggcgct ttcctacctt ggcggcgagg cggcacaggt ggccattggc 120

atatcggaaa agtccgcccg tgtgtttcag ggcatgatcc tgtttttcgt gcttgcttgc 180

gatacgctca ttctttatcg catccgcatt gtgatgaacc gccaggcggg gaaggcctga 240

tctcatggat ttgacacagg caatccttct caccatcgct accgcagcaa caccgctcct 300

gattgcggcc att ggc gaa ctg gtg gtg gaa cgc tcc ggc gtt ctc aat     349
              Gly Glu Leu Val Val Glu Arg Ser Gly Val Leu Asn
              1               5                   10

ctc ggc gtc gag ggc atg agc tgn tgg gcg cgg ttt ccg gct tcg ccg     397
Leu Gly Val Glu Gly Met Ser Xaa Trp Ala Arg Phe Pro Ala Ser Pro
        15                  20                  25

tgg cgc aga tan ccg gct cgg cct ggc tcg gcc ttg ttg ccg ccg ttc     445
Trp Arg Arg Xaa Pro Ala Arg Pro Gly Ser Ala Leu Leu Pro Pro Phe
    30                  35                  40

tgg tcg ggg cgc tgt ttg cgg cag tct tcg gct ttc tta cgc tca ccc     493
```

Trp Ser Gly Arg Cys Leu Arg Gln Ser Ser Ala Phe Leu Arg Ser Pro
45 50 55 60

tcg tca cca atc agg tgg cga ccg gcc ttgcacctgt ctcttg 536
Ser Ser Pro Ile Arg Trp Arg Pro Ala
65

<210> 24
<211> 69
<212> PRT
<213> Brucella melitensis

<400> 24
Gly Glu Leu Val Val Glu Arg Ser Gly Val Leu Asn Leu Gly Val Glu
1 5 10 15

Gly Met Ser Xaa Trp Ala Arg Phe Pro Ala Ser Pro Trp Arg Arg Xaa
20 25 30

Pro Ala Arg Pro Gly Ser Ala Leu Leu Pro Pro Phe Trp Ser Gly Arg
35 40 45

Cys Leu Arg Gln Ser Ser Ala Phe Leu Arg Ser Pro Ser Ser Pro Ile
50 55 60

Arg Trp Arg Pro Ala
65

<210> 25
<211> 190
<212> DNA
<213> Brucella melitensis

<220>
<221> CDS
<222> (3)..(188)

<400> 25
gc ctt gca ctc act att ctg ggc gtc ggc gta tcg gca ttg cct gga 47
Leu Ala Leu Thr Ile Leu Gly Val Gly Val Ser Ala Leu Pro Gly
1 5 10 15

gaa agc ttc gtc ggg ctt ccg ggc gcg cgg ctg aac ccg atc tat att 95
Glu Ser Phe Val Gly Leu Pro Gly Ala Arg Leu Asn Pro Ile Tyr Ile
20 25 30

ccc tat ctg act gat ctt ccg ctc atc ggg cgc ttt ctc ttc ggt cag 143
Pro Tyr Leu Thr Asp Leu Pro Leu Ile Gly Arg Phe Leu Phe Gly Gln
35 40 45

gat ccc atc ttc tat ata tcc cgg ccc cgt gta cta gtc gac gcg tg 190
Asp Pro Ile Phe Tyr Ile Ser Arg Pro Arg Val Leu Val Asp Ala
50 55 60

<210> 26
<211> 62
<212> PRT
<213> Brucella melitensis

<400> 26
Leu Ala Leu Thr Ile Leu Gly Val Gly Val Ser Ala Leu Pro Gly Glu
1 5 10 15

Ser Phe Val Gly Leu Pro Gly Ala Arg Leu Asn Pro Ile Tyr Ile Pro
20 25 30

Tyr Leu Thr Asp Leu Pro Leu Ile Gly Arg Phe Leu Phe Gly Gln Asp
35 40 45

Pro Ile Phe Tyr Ile Ser Arg Pro Arg Val Leu Val Asp Ala
50 55 60

<210> 27
<211> 360
<212> DNA
<213> Brucella melitensis

<220>
<221> CDS
<222> (2)..(358)

<400> 27
g gca ccg cag atc gtt ttg act gtg gtt ttc ttc ttc tgg ccg gca agt 49
Ala Pro Gln Ile Val Leu Thr Val Val Phe Phe Phe Trp Pro Ala Ser
1 5 10 15

cag gca att tat tan tcc ttc atg cgg gaa gac gca ttc ggg ctc aaa 97
Gln Ala Ile Tyr Xaa Ser Phe Met Arg Glu Asp Ala Phe Gly Leu Lys
20 25 30

```
tcc acc ttn gtg gaa ctg gcc aat ttc acc gcc gtc ctt tcg gac ccg     145
Ser Thr Xaa Val Glu Leu Ala Asn Phe Thr Ala Val Leu Ser Asp Pro
        35                  40                  45

aat tat ctc cat tcc gtt cag gtc acg gtc gtt ttc aac gtc ttg acc     193
Asn Tyr Leu His Ser Val Gln Val Thr Val Val Phe Asn Val Leu Thr
    50                  55                  60

gcc ctg ctc gcc atg ggt gtc gcg ctg ctg ctt gca acc gct gcc gac     241
Ala Leu Leu Ala Met Gly Val Ala Leu Leu Leu Ala Thr Ala Ala Asp
65                  70                  75                  80

cgc gtc att cgc ggc cag acc ttc tac cgt act ctt ctg atc tgg ccc     289
Arg Val Ile Arg Gly Gln Thr Phe Tyr Arg Thr Leu Leu Ile Trp Pro
                85                  90                  95

tat gcc gtg gcg cct gct gtc gcg ggc atg ttg tgg ctg ttc atg ttc     337
Tyr Ala Val Ala Pro Ala Val Ala Gly Met Leu Trp Leu Phe Met Phe
            100                 105                 110

aat ccg gcc atg ggc acg ttt gc                                      360
Asn Pro Ala Met Gly Thr Phe
        115


<210> 28
<211> 119
<212> PRT
<213> Brucella melitensis

<400> 28
Ala Pro Gln Ile Val Leu Thr Val Val Phe Phe Phe Trp Pro Ala Ser
1               5                   10                  15

Gln Ala Ile Tyr Xaa Ser Phe Met Arg Glu Asp Ala Phe Gly Leu Lys
            20                  25                  30

Ser Thr Xaa Val Glu Leu Ala Asn Phe Thr Ala Val Leu Ser Asp Pro
        35                  40                  45

Asn Tyr Leu His Ser Val Gln Val Thr Val Val Phe Asn Val Leu Thr
    50                  55                  60

Ala Leu Leu Ala Met Gly Val Ala Leu Leu Leu Ala Thr Ala Ala Asp
65                  70                  75                  80

Arg Val Ile Arg Gly Gln Thr Phe Tyr Arg Thr Leu Leu Ile Trp Pro
                85                  90                  95
```

Tyr Ala Val Ala Pro Ala Val Ala Gly Met Leu Trp Leu Phe Met Phe
100 105 110

Asn Pro Ala Met Gly Thr Phe
115

<210> 29
<211> 375
<212> DNA
<213> Brucella melitensis

<220>
<221> CDS
<222> (1)..(336)

<400> 29
aaa ggc atc gcg gaa ttc ttc aat ttc ctc tcg cag agc aag att cag 48
Lys Gly Ile Ala Glu Phe Phe Asn Phe Leu Ser Gln Ser Lys Ile Gln
1 5 10 15

gtc aag ctg cat gaa aaa tcc ggc tat ctg ccg gtg acg ctt gca gcc 96
Val Lys Leu His Glu Lys Ser Gly Tyr Leu Pro Val Thr Leu Ala Ala
20 25 30

tat gag gaa acc aag aag tcg gat ttc tat gaa aag aat ccg ggc cgc 144
Tyr Glu Glu Thr Lys Lys Ser Asp Phe Tyr Glu Lys Asn Pro Gly Arg
35 40 45

gaa acg ccg atc ctg caa atg atg ggc aag gag ccg acg gaa aat tcc 192
Glu Thr Pro Ile Leu Gln Met Met Gly Lys Glu Pro Thr Glu Asn Ser
50 55 60

aag ggt gtg cgc ctc gtc aac ctg ccg cag gtt cgc gac att ctg aat 240
Lys Gly Val Arg Leu Val Asn Leu Pro Gln Val Arg Asp Ile Leu Asn
65 70 75 80

gaa gag ttt gaa gcc atg ctg ggc ggc aaa cag gac gcg aag acc gca 288
Glu Glu Phe Glu Ala Met Leu Gly Gly Lys Gln Asp Ala Lys Thr Ala
85 90 95

ctc gac aac gcc gtc aag cgc ggc aac gca gca atc gcc gca gct caa 336
Leu Asp Asn Ala Val Lys Arg Gly Asn Ala Ala Ile Ala Ala Ala Gln
100 105 110

taatccatca tatcgagaca atgccgccaa ccctttccg 375

<210> 30
<211> 112
<212> PRT
<213> Brucella melitensis

<400> 30
Lys Gly Ile Ala Glu Phe Phe Asn Phe Leu Ser Gln Ser Lys Ile Gln
1   5   10   15

Val Lys Leu His Glu Lys Ser Gly Tyr Leu Pro Val Thr Leu Ala Ala
            20   25   30

Tyr Glu Glu Thr Lys Lys Ser Asp Phe Tyr Glu Lys Asn Pro Gly Arg
        35   40   45

Glu Thr Pro Ile Leu Gln Met Met Gly Lys Glu Pro Thr Glu Asn Ser
    50   55   60

Lys Gly Val Arg Leu Val Asn Leu Pro Gln Val Arg Asp Ile Leu Asn
65   70   75   80

Glu Glu Phe Glu Ala Met Leu Gly Gly Lys Gln Asp Ala Lys Thr Ala
                85   90   95

Leu Asp Asn Ala Val Lys Arg Gly Asn Ala Ala Ile Ala Ala Ala Gln
            100   105   110

<210> 31
<211> 249
<212> DNA
<213> Brucella melitensis

<400> 31
gttctggacn atgcnggaga cgggggtccn agccgngggg catccaccat caccatgcag 60
atggtgaaga acctgttntt gtggaatggg cgctcctatc tgcgcaaagg gctggaattt 120
ccgctggcgt tgattctgat caagagacag gtccagancg aggcctagct gatgccagtc 180
caccccnccg tggctgcaaa attggccatc ctccgccatc atcaccgaat tgaccagaac 240
cggcgcgat 249

<210> 32
<211> 333
<212> DNA
<213> Brucella melitensis

<400> 32
gcgcagaggc actgcatgcg agcggttgcg gataccсttg atcgcagtca gtgtggcaat 60
ggcttccccg gcaggcaggt cgcacaggcc gtgaagatcg agcgcaccgc ctgccagtgc 120
ctcgcttaac gccagaagcg ttctctgctt gggccgcgaa agcccggcca aacgccacgc 180
ctcctccccg cccgcgatat aggcttccgg cgtcagcgga ttgatcacct gtttcaaccg 240
cgcccagatg gcggcggcgc tggcggtcga aacctgctgc gcgacgacga tggaagccag 300
actttcaaat ccgggttcgg accggcgcag agg 333

<210> 33
<211> 99
<212> DNA
<213> Brucella melitensis

<400> 33
cccagttctt gttcttgaaa gtcacagatc tcattgaatc atccсttttc cgtatcgcgt 60
ctggtgatcg cgcagttcaa ttgacaggga atatagcgg 99

<210> 34
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer
sequence

<400> 34
tacctacaac ctcaagct 18

<210> 35
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer
sequence

<400> 35
tacccattct aaccaagc 18